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WANDA MONTGOMERY

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CLONED GLUCAGON-LIKE PEPTIDE 2 RECEPTORS

FIELD OF THE INVENTION

The invention is in the field of molecular biology. It relates, more particularly, to cloned glucagon-like peptide 2 receptors and their use in drug screening and related applications.

BACKGROUND TO THE INVENTION

Glucagon-like peptide-2 (GLP-2) is a 33 amino acid peptide, which is expressed in a tissue determined fashion from the pleotropic glucagon gene and is highly related in terms of amino acid sequence to glucagon and Glucagon-like peptide-1 (GLP-1). Mammalian forms of GLP-2 are highly conserved: for example, the human and degu (a south American rodent) forms differ by one and three amino acids respectively from rat GLP-2. Recently it was demonstrated that GLP-2 is an intestinotrophic peptide hormone; when given exogenously, GLP-2 can produce a marked increase in the proliferation of small intestinal epithelium of the test mice (Drucker et al, (1996) PNAS, 93:7911-7961). More recently, GLP-2 has been shown to increase D-Glucose maximal transport rate across the intestinal basolateral membrane. (Cheeseman and Tseng: American Journal of Physiology (1996) 271:G477-G482)

To accelerate research into gastrointestinal biology and development of drugs useful in the treatment of gastrointestinal disorders, it would be useful to provide the receptor through which the effects of GLP-2 are mediated.

SUMMARY OF THE INVENTION

The GLP-2 receptor has now been cloned and characterized. Accordingly, the present invention provides an isolated polynucleotide encoding a GLP-2 receptor. In aspects of the invention, polynucleotide coding for the GLP-2 receptor is utilized for expression to obtain functional receptor protein and for further gene cloning to identify structurally related receptor proteins. In related aspects of the invention, anti-sense versions of GLP-2 receptor-encoding polynucleotides and fragments thereof are obtained and utilized to regulate GLP-2 receptor expression.

In another of its aspects, the invention provides GLP-2 receptor as a product of recombinant production in a cellular host. In related aspects, there are provided recombinant host cells that express GLP-2 receptor, as well as receptor-bearing membranes derived from such cells, and expression constructs in which polynucleotide coding for the GLP-2 receptor is linked to expression controls functional in the selected host cell.

In another of its aspects, the GLP-2 receptor is utilized in a chemicals screening program to identify GLP-2 receptor ligands. This method comprises the steps of incubating the candidate ligand with an GLP-2 receptor-producing cell of the present invention, or with a membrane preparation derived therefrom, and then assessing the interaction by determining receptor/candidate ligand binding.

In another of its aspects, the invention provides antibodies directed to the GLP-2 receptor, for use for example in diagnostic procedures.

The invention is further described with reference to the following drawings in which:

BRIEF REFERENCE TO THE DRAWINGS

Figure 1 discloses a cDNA sequence (SEQ ID No. 1), nucleotides 17 to 1546 of which encode the rat GLP-2 receptor;

Figure 2 discloses the expression product (SEQ ID No. 2) of the cDNA of Figure 1; and

Figure 3 illustrates the relative binding affinities of a GLP-2 peptide and a GLP-1 peptide for the receptor encoded by SEQ ID No. 1.

DETAILED DESCRIPTION AND PREFERRED EMBODIMENTS

The invention relates in one respect to polynucleotides that code for GLP-2 receptors. Such polynucleotides may be in the form of RNA, or in the form of DNA including cDNA, genomic DNA and synthetic DNA. The GLP-2 receptors are characterized by structural features common to the G-protein coupled receptor class, including seven transmembrane regions, and by the functional properties of binding GLP-2 peptide selectively relative to GLP-1 peptide and, when expressed functionally in a host cell, of responding to GLP-2 binding by signal transduction.

The activity of a G-protein coupled receptor such as a GLP-2 receptor can be measured using any of a variety of appropriate functional assays in which activation of the receptor results in an observable change in the level of some second messenger system, such as adenylate cyclase, calcium mobilization, inositol phospholipid hydrolysis or guanylyl cyclase.

In one embodiment of the invention, the GLP-2 receptor is encoded by the polynucleotide sequence of SEQ ID No. 1. This particular GLP-2 receptor-encoding polynucleotide, also referred to as the WBR gene, is a cDNA of rat origin. The expression product of this polynucleotide incorporates the mature form of the GLP-2 receptor, and may incorporate a secretion signal that is removed before membrane integration of the mature GLP-2 receptor product. The expressed GLP-2 receptor product (Figure 2, SEQ ID No.2) is characterized structurally as a single 509 amino acid polypeptide chain having a predicted molecular weight of 59kDa. With respect to structural domains of this GLP-2 receptor, hydropathy analysis and sequence alignment with related members of this sub-family of G protein coupled receptors indicates seven putative transmembrane domains, one spanning residues 140-162 inclusive (TM I), another spanning residues 170-189 (TM II), a third spanning residues 221-244 (TM III), a fourth spanning residues 259-280 (TM IV), a fifth spanning 298-321 (TM V), a sixth spanning 345-364 (TM VI) and a seventh spanning 381-400 (TM VII). Based on this assignment, it is likely that this GLP-2 receptor, in its natural membrane-bound form, consists of a an N-terminal extracellular domain, followed by a hydrophobic region containing seven transmembrane domains and an intracellular 401-509 amino acid

C-terminal domain. The protein exhibits the highest degree of homology to the rat GLP-1 receptor with 38% identity at the amino acid level. Yet another aspect of the invention is amino acid sequences corresponding to any of the above domains, and nucleotide sequences which encode these amino acid sequences.

In another embodiment, the invention provides GLP-2 receptor polynucleotide sequences as a tool useful to identify structurally related polynucleotides. At low stringency hybridization conditions, for instance, polynucleotide libraries can be probed to identify genes that are at least about 40% homologous to the GLP-2 receptor gene. To facilitate isolation of WBR gene homologs that are also GLP-2 receptor-encoding, stringency conditions are desirably enhanced to identify homologs having at least 80% sequence identity at the polynucleotide level to WBR. More desirably the WBR gene homologs are 90% identical, and most desirably they have at least 95% sequence identity when compared to WBR. Preferably, the isolated WBR homologs are characterized in that (1) they can be amplified using the PCR primers of SEQ ID No.3 and SEQ ID No. 4 and (2) they bind to the probe of SEQ ID No.5.

In order to isolate GLP-2 receptor encoding homologs of the WBR gene, it is desirable but not necessary to use libraries of fetal or mature hypothalamal, jejunal or hindbrain tissue obtained from the vertebrate species targeted for receptor isolation. The invention accordingly includes not only the WBR but structural homologs thereof and particularly those that code for proteins having GLP-2 receptor properties. Thus, the invention provides polynucleotides that encode GLP-2 receptors, including rat GLP-2 receptor and vertebrate homologs, particularly mammalian homologs thereof including human homologs thereof, as well as synthetic variants of these.

It will be appreciated that such homologs can also be identified in libraries by screening with fragments of the WBR gene, which incorporate at least 15 nucleotides, and preferably at least ²⁵ nucleotides. With reference to SEQ ID No. 1 and the nucleotide numbering appearing thereon, such nucleotide fragments include those corresponding in sequence to the extracellular GLP-2 binding domain, and the transmembrane regions.

Technically, the identification of WBR-related genes can be achieved by applying standard hybridization or amplification techniques to a tissue-derived polynucleotide library. A wide variety of

such libraries are commercially available. Where construction of a cDNA library is necessary, established techniques are applied. For example, isolation of such a WBR homolog typically will entail extraction of total messenger RNA from a fresh source of tissue, such as hypothalamic, jejunal or hindbrain tissue, preferably hypothalamic tissue, followed by conversion of message to cDNA and formation of a library in for example a bacterial plasmid, more typically a bacteriophage. Such bacteriophage harbouring fragments of the DNA are typically grown by plating on a lawn of susceptible *E. coli* bacteria, such that individual phage plaques or colonies can be isolated. The DNA carried by the phage colony is then typically immobilized on a nitro-cellulose or nylon-based hybridization membrane, and then hybridized, under carefully controlled conditions, to a radioactively (or otherwise) labelled probe sequence to identify the particular phage colony carrying the fragment of DNA of particular interest, in this case a WBR homolog. The phage carrying the particular gene of interest is then purified away from all other phages from the library, in order that the foreign gene may be more easily characterized. Typically, the gene or a portion thereof is subcloned into a plasmidic vector for convenience, especially with respect to the full determination of its DNA sequence.

As an alternative to obtaining GLP-2 encoding DNA directly as a DNA insert from an available or a constructed cDNA library, in light of the present disclosure it can be synthesized *de novo* using established techniques of gene synthesis. Because of the length of the GLP-2 receptor-encoding DNA of SEQ ID NO 1, application of automated synthesis may require staged gene construction, in which regions of the gene up to about 300 nucleotides in length are synthesized individually and then ligated in correct succession for final assembly. Individually synthesized gene regions can be amplified by PCR. The application of automated synthesis may typically be applied by synthesizing specific regions or fragments of the gene and ligating them, usually via designed overlaps, in correct succession to form the final gene sequence. In this case, the longer the oligonucleotide building blocks, the fewer will be the ligations needed, resulting in greater ease of assembly.

The application of automated gene synthesis techniques provides an opportunity for generating sequence variants of the naturally occurring GLP-2 receptor gene. It will be appreciated, for example, that polynucleotides coding for the GLP-2 receptor herein described can be generated by substituting synonymous codons for those represented in the naturally occurring polynucleotide sequences herein

provided. In addition, polynucleotides coding for synthetic variants of the GLP-2 receptor herein provided can be generated which incorporate from 1 to 20, e.g., from 1 to 5, amino acid substitutions, or deletions or additions. Since it will be desirable typically to retain the natural ligand binding profile of the receptor for screening purposes, it is desirable to limit amino acid substitutions, for example to the so-called conservative replacements in which amino acids of like charge are substituted, and to limit substitutions to those sites less critical for receptor activity.

Having obtained GLP-2 receptor encoding polynucleotide, GLP-2 receptor can be produced in a number of ways, including in vitro transcription and via incorporation of the DNA into a suitable expression vector and expression in the appropriate host, for example a bacteria such as E.coli, yeast or a mammalian cell. A variety of gene expression systems have been adapted for use with these hosts and are now commercially available, and any one of these systems can be selected to drive expression of the GLP-2 receptor-encoding DNA. Expression vectors may be selected to provide transformed cell lines that express the receptor-encoding DNA either transiently or in a stable manner. For transient expression, host cells are typically transformed with an expression vector harbouring an origin of replication functional in a mammalian cell. For stable expression, such replication origins are unnecessary, but the vectors will typically harbour a gene coding for a product that confers on the transformants a survival advantage, to enable their selection such as a gene coding for neomycin resistance in which case the transformants are plated in medium supplemented with neomycin.

These expression systems, available typically but not exclusively in the form of plasmidic vectors, incorporate expression cassettes the functional components of which include DNA constituting expression controlling sequences, which are host-recognized and enable expression of the receptor-encoding DNA when linked 5' thereof. The systems further incorporate DNA sequences which terminate expression when linked 3' of the receptor-encoding region. Thus, for expression in the selected mammalian cell host, there is generated a recombinant DNA expression construct in which the receptor-encoding DNA is linked with expression controlling DNA sequences recognized by the host, and which include a region 5' of the receptor-encoding DNA to drive expression, and a 3' region to terminate expression.

Included among the various recombinant DNA expression systems that can be used to achieve mammalian cell expression of the receptor-encoding DNA are those that exploit promoters of viruses that infect mammalian cells, such as the promoter from the cytomegalovirus (CMV), the Rous sarcoma virus (RSV), simian virus (SV40), murine mammary tumor virus (MMTV) and others. Also useful to drive expression are promoters such as the LTR of retroviruses, insect cell promoters such as those regulated by temperature, and isolated from *Drosophila*, as well as mammalian gene promoters such as those regulated by heavy metals i.e. the metallothionein gene promoter, and other steroid-inducible promoters.

In another of its aspects, the invention provides cells or membranes derived therefrom which are adapted by genetic alteration for use, for example, in identifying GLP-2 receptor ligands. In preferred embodiments, such cells are adapted genetically by the insertion of polynucleotide coding for a GLP-2 receptor. In particularly preferred embodiments, such cells incorporate a recombinant DNA molecule, e.g. an expression construct/vector, in which DNA coding for the GLP-2 receptor and expression controlling elements functional in the host are linked operably to drive expression of the DNA. For incorporation of receptor into cell plasma membranes, the vector can be designed to provide a suitable heterologous signal peptide sequence if the naturally occurring signal peptide is not encoded within the receptor DNA.

Suitable GLP-2 producing cells include the Chinese hamster ovary (CHO) cells for example of K1 lineage (ATCC CCL 61) including the Pro5 variant (ATCC CRL 1281); the fibroblast-like cells derived from SV40-transformed African Green monkey kidney of the CV-1 lineage (ATCC CCL 70), of the COS-1 lineage (ATCC CRL 1650) and of the COS-7 lineage (ATCC CRL 1651); murine L-cells, murine 3T3 cells (ATCC CRL 1658), murine C127 cells, human embryonic kidney cells of the 293 lineage (ATCC CRL 1573), human carcinoma cells including those of the HeLa lineage (ATCC CCL 2), and neuroblastoma cells of the lines IMR-32 (ATCC CCL 127), SK-N-MC (ATCC HTB 10) and SK-N-SH (ATCC HTB 11).

For use in ligand screening assays, cell lines expressing the receptor-encoding DNA can be stored frozen for later use. Such assays may be performed either with intact cells, or with membrane

preparations derived from such cells. The membrane preparations typically provide a more convenient substrate for the ligand binding experiments, and are therefore preferred as binding substrates. To prepare membrane preparations for screening purpose, i.e., ligand binding experiments, frozen intact cells are homogenized while in cold water suspension and a membrane pellet is collected after centrifugation. The pellet is then washed in cold water, and dialyzed to remove any endogenous GLP-2 receptor ligands that would otherwise compete for binding in the assays. The dialyzed membranes may then be used as such, or after storage in lyophilized form, in the ligand binding assays.

The binding of a candidate ligand to a selected GLP-2 receptor of the invention is performed typically using a predetermined amount of cell-derived membrane (measured for example by protein determination), generally from about 25ug to 100ug. Generally, competitive binding assays will be useful to evaluate the affinity of a test compound relative to GLP-2. This competitive binding assay is performed by incubating the membrane preparation with radiolabelled GLP-2 peptide, for example [³H]-GLP-2 or a radioiodinated GLP-2 analog, in the presence of unlabelled test compound added at varying concentrations. Following incubation, either displaced or bound radiolabelled GLP-2 can be recovered and measured, to determine the relative binding affinities of the test compound and GLP-2 for the GLP-2 receptor used as substrate. In this way, the affinities of various compounds for the GLP-2 receptor can be measured.

Alternatively, intact, fresh cells, harvested about two days following after transient transfection or after about the same period following fresh plating of stably transfected cells, can be used for ligand binding assays by the same methods as used for membrane preparations. In this case, the cells must be harvested by more gentle centrifugation so as not to damage them, and all washing must be done in a buffered medium.

As an alternative to using cells that express receptor-encoding DNA, ligand characterization may also be performed using cells for example *Xenopus* oocytes, that yield functional membrane-bound receptor following introduction of messenger RNA coding for the GLP-2 receptor. In this case, the GLP-2 receptor gene of the invention is typically subcloned into a plasmidic vector such that the introduced gene may be easily transcribed into RNA via an adjacent RNA transcription promoter supplied by the

plasmidic vector, for example the T3 or T7 bacteriophage promoters. RNA is then transcribed from the inserted gene in vitro, and can then be injected into *Xenopus* oocytes. Each oocyte is a single cell, but is large enough to be penetrated by a fine-tipped microneedle without causing irreparable damage. Following the injection of nL volumes of an RNA solution, the oocytes are left to incubate for up to several days, whereupon the oocytes are tested for the ability to respond to a particular ligand molecule supplied in a bathing solution.

Candidate GLP-2 receptor ligands can vary widely in structure, and include proteins which are highly related to GLP-2 itself in terms of amino acid sequence. For instance, the peptides disclosed in co-pending United States patent applications 08/422,540 and 08/631,273, incorporated herein by reference, may usefully be screened for GLP-2 receptor binding activity. Furthermore, the advent of high throughput screening makes feasible the screening of a chemical library containing hundreds or thousands of test compounds for GLP-2 receptor binding activity.

In addition to using the receptor-encoding DNA to construct cell lines useful for ligand screening, expression of the DNA can according to another aspect of the invention be performed to produce fragments of the receptor in soluble form, for structure investigation, to raise antibodies and for other experimental uses. It is expected that the portion of the GLP-2 receptor responsible for binding a ligand molecule resides on the outside of the cell, i.e., is extracellular. It is therefore desirable in the first instance to facilitate the characterization of the receptor-ligand interaction by providing this extracellular ligand-binding domain in quantity and in isolated form, i.e., free from the remainder of the receptor.

To accomplish this, the full-length GLP-2 receptor-encoding DNA may be modified by site-directed mutagenesis, so as to introduce a translational stop codon into the extracellular N-terminal region, immediately before the sequence encoding the first transmembrane domain (TM1), i.e., before residue 140 of SEQ ID No. 2. Since there will no longer be produced any transmembrane domain(s) to "anchor" the receptor into the membrane, expression of the modified gene will result in the secretion, in soluble form, of only the extracellular ligand-binding domain. Standard ligand-binding assays may then be performed to ascertain the degree of binding of a candidate compound to the extracellular domain

so produced. It may of course be necessary, using site-directed mutagenesis, to produce several different versions of the extracellular regions, in order to optimize the degree of ligand binding to the isolated domains.

It will be appreciated that the production of such extracellular ligand binding domains may be accomplished in a variety of host cells. Mammalian cells such as CHO cells may be used for this purpose, the expression typically being driven by an expression promoter capable of high-level expression, for example the CMV (cytomegalovirus) promoter. Alternately, non-mammalian cells, such as insect Sf9 (*Spodoptera frugiperda*) cells may be used, with the expression typically being driven by expression promoters of the baculovirus, for example the strong, late polyhedrin protein promoter. Filamentous fungal expression systems may also be used to secrete large quantities of such extracellular domains of the GLP-2 receptor. *Aspergillus nidulans*, for example, with the expression being driven by the alcA promoter, would constitute such an acceptable system. In addition to such expression hosts, it will be further appreciated that any prokaryotic or other eukaryotic expression system capable of expressing heterologous genes or gene fragments, whether intracellularly or extracellularly would be similarly acceptable.

The availability of isolated extracellular ligand-binding domains of the receptor protein makes it feasible to determine the 3-dimensional structures of these ligand-binding regions, with or without a candidate ligand complexed thereto, by a combination of X-ray crystallographic and advanced 2D-NMR techniques. In this way, additional new candidate compounds, predicted to have the required interactions with the 3-dimensional receptor structure, can be specifically designed and tested.

With large domains, crystallography is the method of choice for structure determination of both the domain in isolation, and of the co-complex with the natural ligand (or an appropriate antagonist or agonist molecule). If a particular domain can be made small enough, for example approx. 100-130 amino acids in length, then the powerful technique of 2-D NMR can also be applied to structure determination. This enables not only the determination of the domain structure, but also provides dynamic information about the drug-receptor interaction.

For use particularly in detecting the presence and/ or location, for example in intestinal tissue, the present invention also provides, in another of its aspects, labelled antibody to a GLP-2 receptor. To raise such antibodies, there may be used as immunogen either the intact, soluble receptor or an immunogenic fragment thereof, produced in a microbial or mammalian cell host as described above or by standard peptide synthesis techniques. Regions of the GLP-2 receptor particularly suitable for use as immunogenic fragments include those corresponding in sequence to an extracellular region of the receptor, or a portion of the extracellular region, such as peptides consisting of 10 or more amino acids of the 401-509 region of SEQ ID No. 2.

Antibodies to the desired GLP-2 receptor or fragment immunogen are available, for polyclonal antibody production, from the blood of an animal that has been immunized with the immunogen. Alternatively, for monoclonal antibody production, immunocytes such as splenocytes can be recovered from the immunized animal and fused, using hybridoma technology, to a myeloma cells. The fusion products are then screened by culturing in a selection medium, and cells producing antibody are recovered for continuous growth, and antibody recovery. Recovered antibody can then be coupled covalently to a detectable label, such as a radiolabel, enzyme label, luminescent label or the like, using linker technology established for this purpose.

Animal model systems which elucidate the physiological and behavioral roles of the GLP-2 receptor are produced by creating transgenic animals in which the activity of the GLP-2 receptor is either increased or decreased, or the amino acid sequence of the expressed GLP-2 receptor is altered, by a variety of techniques. Examples of these techniques include, but are not limited to:

- 1) Insertion of normal or mutant versions of DNA encoding a GLP-2 receptor, by microinjection, electroporation, retroviral transfection or other means well known to those skilled in the art, into appropriate fertilized embryos in order to produce a transgenic animal or
- 2) Homologous recombination of mutant or normal, human or animal versions of these genes with the native gene locus in transgenic animals to alter the regulation of expression or the structure of these GLP-2 receptor sequences. The technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and so is useful for producing an animal that cannot express native GLP-2 receptors but does express, for example, an inserted mutant GLP-2

receptor, which has replaced the native GLP-2 receptor in the animal's genome by recombination, resulting in underexpression of the transporter. Microinjection adds genes to the genome, but does not remove them, and so is useful for producing an animal which expresses its own and added GLP-2 receptors, resulting in overexpression of the GLP-2 receptors.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium. DNA or cDNA encoding a GLP-2 receptor is cesiumchloride purified from a vector by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the transgene. Alternatively or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the transgene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a piper puller) and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only methods for inserting DNA into the egg cell, and is used here only for exemplary purposes.

Example 1 Isolation of the GLP-2 receptor

PCR-assisted cloning of partial rat and mouse GLP-2 receptor cDNAs

Rat Neonate Intestine cDNA library (Stratagene, Cat. 936508) and Mouse Jejunum first strand cDNA was prepared. Degenerate primers M-2F/S (SEQ ID No. 3) and M-7R/S (SEQ ID No. 4) were used to amplify a partial fragment of the rat GLP-2 receptor from the Rat Neonate Intestine cDNA library and of the mouse GLP-2 receptor from Mouse Jejunum template. The protocol is described below:

Degenerate PCR :

6 ul 10x VENT buffer from New England Biolabs
6 ul 2.5uM each stock dATP, dCTP, dGTP and dTTP
4 ul rat neonate intestine cDNA (1:10 dilution)
3 ul 25uM primer [5'-TTTTTCTAGAASRTSATSTACACNGTSGGCTAC-3'] (SEQ ID No. 3)
3 ul 25uM primer [5'-TTTTCTCGAGCCARCCASSWRTARTTGGC-3'] (SEQ ID No. 4)
2 ul (10 units) Amplitaq DNA polymerase (Perkin Elmer)
36 ul ddH₂O
Reaction conditions: 35 cycles at 94 C, 2 min.; 94 C, 1 min.; 53 C, 30 sec.; 72 C, 1 min.
The predominant PCR product was a 303 base pair (bp) DNA fragment. 30 ul samples of the above PCR were purified using the QIAGEN PCR purification kit and eluted in 30 ul ddH₂O.

Reamplification PCR:

6 ul 10x VENT buffer from New England Biolabs
6 ul 2.5uM each stock dATP, dCTP, dGTP and dTTP
4 ul above purified PCR template
3 ul 25uM primer [5'-TTTTTCTAGAASRTSATSTACACNGTSGGCTAC-3']
3 ul 25uM primer [5'-TTTTCTCGAGCCARCCASSWRTARTTGGC-3']
2 ul (10 units) Amplitaq DNA polymerase (Perkin Elmer)
36 ul ddH₂O
Reaction conditions: 31 cycles at 94 C, 2 min.; 94 C, 1 min.; 53 C, 30 sec.; 72 C, 1 min.
The predominant product at 303 base pair (bp) was cut out and purified using QIAGEN QIAquick gel purification protocol into 30 ul ddH₂O.

Next, double digest (XbaI and XhoI) was done on the entire reamplified PCR reaction as follows:

28 ul DNA
16 ul 10X One-Phor-All buffer (Pharmacia)
2 ul (40 units) XbaI enzyme (Pharmacia)
2 ul (40 units) XhoI enzyme (Pharmacia)
30 ul ddH₂O

The samples were digested 4 hours in 37 C water block heater, brought up to 100 ul volume with ddH₂O (sterile) and purified by (1) equal amount (100ul) chloroform extraction, (2) Weekend precipitation with 2 vol EtOH/10 vol 3M sodium acetate; (3) 1x wash with 70% EtOH, and (4) resuspension in 10 ul 1x TE (pH 8.0).

pBluescript clone 5HT1F#9 was next digested with XbaI and XhoI as follows:

10 ul DNA (pBluescript clone 5HT1F#9)
5 ul 10X NEBuffer 2 (New England Biolabs)
3 ul (1:20 dilution=3 units) Xba I (New England Biolabs)
3 ul (1:20 dilution=3 units) Xho I (New England Biolabs)
5 ul (10x) BSA (New England Biolabs)
24 ul ddH₂O

The sample was digested for 3 hours in 37 C water block heater, heat-inactivated at 65 C for 20 min and purified using GeneCleanII kit from BIO 101. Aliquots of the PCR reactions were cloned into the above pBluescript plasmid vector using T4 DNA ligase kit (New England Biolabs) and transformed into Epicurian Coli XL-2 Blue MRF' Ultracompetent cells (Stratagene). The transformation was plated onto 2xYT + AMP plates and single colonies were picked. DNA minipreps were made using QIAGEN QIA-prep 8 miniprep kit and the sequences of the fragments were determined using ABI system. Novel sequences were identified containing a partial fragment of the rat and mouse GLP-2 receptor sequence.

Cloning of cDNA with complete GLP-2 receptor coding region was achieved as follows:
First, cDNA libraries from the following three tissues were used for screening,

1. Rat Hypothalamus (RHT)
2. Rat Hind Brain (RHB)
3. Rat Duodenum and Jejunum (RDJ)

The three cDNA libraries were prepared by priming with random primer and subcloning unidirectionally into Hind III and NotI sites of pcDNA3.

Next, the three cDNA libraries were homology screened by a degenerate oligo C4-4 [5'-AACTACATCCACMKGMAYC TGTTYVYGTCBTTCATSC-3'] (SEQ ID No.5) by colony lifts and filter hybridization. The following hybridization conditions were employed:
5X SSPE (1X SSPE is 0.18M NaCl, 10mM NaH₂PO₄ (pH 7.4), 10mM EDTA (pH 7.4))
5X Denharts solution (1% Ficoll, 1% Polyvinylpyrrolidone, 1% BSA); 25 mg/ml salmon sperm DNA.

Filters were hybridized at 50°C overnight. Then the filters were washed 2 times in 2X SSPE and 1% SDS at room temperature for 30 min, then 2 times in 2X SSPE and 1% SDS at 50°C for 20 min per wash and finally two times in 1X SSPE and 0.5% SDS. Positive clones were identified by autoradiography. A plug of 1 cm² surrounding the positive clone was removed from the plate and placed in 1 ml of 2x YT +20% Glycerol, vortexed and was frozen at -80°C.

Plasmid DNA from positive plugs was prepared as follows: 100 ml of bacterial culture of each positive plug was grown on an agar plate. The bacterial cells were scraped and resuspended in 1 ml of 2xYT medium+20% Glycerol. Bacterial pellet from the 250 ml of bacterial resuspension was resuspended in 150 ml of Solution I (50mM Glucose, 10mM Tris-HCl, 1mM EDTA), lyse in Solution II (0.2M NaOH, 1% SDS), neutralized with ice cold Solution III (Potassium acetate; 4 vol. of 5M potassium acetate + 1 vol. of 10M acetic acid). After pelleting bacterial DNA, 340 ml isopropanol was added to the supernatant. This was centrifuged at max for 15 min. The pellet was resuspended in TE + 20 mg/ml RNase, incubated at 37 C for 30 minutes and precipitated with isopropanol + 0.2M potassium acetate. After centrifugation, the pellet was washed with 70% alcohol, allowed to air dry and resuspended in TE.

Plasmid DNA from 2777-clone pools of rat hypothalamus cDNA library RHT cDNA library was next exploited as follows: Two primers were designed from an area of the PCR-cloned GLP-2 receptor cDNA sequence that did not have identity to known receptors of the gene family. The two primers P23-R1 and P23-F1 amplified a 196 bp fragment only from novel clone DNA but not with GLP-1 receptor cDNA or PACAP receptor cDNA. The Expand™ PCR system from Boehringer Mannheim (Cat. 1681-842) was used under the following conditions:

2 ul of 10x Expand™ Buffer 1

2.8 ul of 2.5mM dNTP mix

0.6 ul of primer P23-R1 [5'-TCATCTCCCTCTTCTTGGCTCTTAC-3'] (SEQ ID No.6)

0.6 ul of primer P23-F1 [5'-TCTGACAGATATGACATCCATCCAC-3'] (SEQ ID No.7)

0.3 ul of Expand PCR enzyme (1unit)

12.7 ul water

1 ul DNA

Reaction conditions: 32 cycles at 93°C, 40 sec; 58°C, 40 sec; 68°C, 40 sec

DNAs from each positive plug or pool of 2777-clone pools were amplified with specific primers P23-F1 and P23-R1 under the conditions specified above. Out of 1057 C4-4 hybridization-positive plugs and 884 2777-clone pools only five template sources amplified a 196 bp PCR product. These were: (1) Plug 334, (2) Plug 780, (3) RHT pool 233, (4) RHT pool 440, and (5) RHT pool 587.

Amplification of GLP-2R cDNA from the five positive templates was then performed. By using one specific primer (P23-R1 or P23-F1) and one primer based on pcDNA3 vector (Invitrogen) sequence (830F or 1186R), the GLP-2R cDNA insert was directly amplified from clonally impure plugs or 2777-clone pools. The sequences of the vector primers were as follows:

830F: [5'-AACCCACTGCTTAC-3']

1186R: [5'-CCCAGAATAGAATGACACC-3']

The PCR was done under the following conditions using Expand™ PCR system from Boehringer Mannheim (Catalogue no. 1681-842).

2 ul of 10x Expand™ Buffer 1

2.8 ul of 2.5mM dNTP mix

0.6 ul of Primer 1

0.6 ul of Primer 2

0.3 ul of Enzyme (1 unit)

12.7 ul water

1 ul DNA

Reaction conditions: 32 cycles at 93°C, 45 sec; 50°C, 45 sec; 68°C, 1 min.

The most prominent band was reamplified, purified and sequenced. Based on the amplified sequence obtained, additional primers were designed and new sequencing carried out. In this manner the complete sequences of the GLP-2R cDNA inserts in all five sources of clones were determined. Sequence analysis showed that only pool RHT 440 and pool RHT 587 contain clones with complete coding sequence of GLP2-R and that the two clones were identical (derived from the same cDNA clone).

Because of difficulty in clonally purifying the GLP-2 receptor cDNA clone from the RHT 440 or RHT 587 cDNA library pools, the cDNA was amplified and recloned into pcDNA3. Based on the sequence obtained from RHT 440 and RHT 587 two primers were designed adjacent to the 5' and 3' ends of the coding region.

WBR-C5: [5' -CAGGGGCCGGTACCTCTCCACTCC-3']

WBR-C3: [5' -TTGGGTCCTCGAGTGGCCAAGCTGCG-3']

The two primers were used to amplify a DNA fragment of approximately 1525 bp fragment under the following PCR conditions using Expand™ PCR system from Boehringer Mannheim (Catalogue no. 1681-842).

10 ul of 10x Expand™ PCR Buffer 1

14 ul of 2.5mM dNTP mix

3.0 ul of Primer 1 (10 uM)

3.0 ul of Primer 2 (10 uM)

1.5 ul of Enzyme (5 units)

63.5 ul water

5 ul DNA

Reaction conditions: 5 cycles (93°C, 1 Min; 72°C, 40s; 60°C, 45 sec; 68°C, 2 min) and 25 cycles (93°C, 1 Min; 72°C, 1min; 68°C, 2 min).

The amplified product was subcloned into KpnI and XhoI sites of pcDNA3 vector (Invitrogen). Plasmid DNA was prepared using the method described above.

Example 2 Ligand Binding Assay

Cos-1 cells were transfected as described in Analytical Biochemistry, 218:460-463(1994) with cloned rat 587- Receptor/GLP-1 Receptor. Solutions used were as follows:

RSC in RPMI 1640 (49ml RPMI + 1ml FCS + 50ul chloroquine, 100mM).

DEAE/RSC Solution: 18.4ml RSC +1.6ml DEAE/Dextran (10mg/ml).

The assay procedure entailed the following:

- a) six ml of RSC was added to 50ml tubes, 50µg cloned 587 (1ug/ml)/ 40ul GLP- 1 receptor (1.2ug/ml) was added to corresponding tubes and incubate at 37C.
- b) six ml of DEAE/RSC solution was added to each tube and incubate at 37C for 2 min.
- c) 1.5 ml cos-1 cells suspensions (5.5 millions cells) added to each tube and incubate for 1hr and 45 min at 37C incubator.
- d) Following incubation sample spinned for 5 min, washed with DMEM/F12 + 10% FBS twice, pellet resuspended in 12.5 ml DMEM/F12 + 10% FBS media.
- e) One ml of cell suspension (step d) added to each well of 6 well plates coated with poly- D-lysine (from Collaborative Biomedical), containing 3ml of media, (0.45 million cells/well).
- f) Plates incubated at 37C for 3 days.

2) Treatment of Transfected Cos-1 cells with GLP-1/GLP-2 analog was done as follows:

Solutions : DMEM/F12 (SFM)+ IBMX (3-isobutyl-1-methylxanthin) 0.85mM +0.1% ascorbic acid and 10um pargylin (all solutions purchased from Sigma). Media prepared fresh on day of use.

Assay Procedure: The culture media of each well (transfected ,6 well plates , cells) was removed, and the wells were washed once with SFM media. Then 2 ml of SFM + IBMX media was added to each well and plates were incubated at 37C for 10 min. Following incubation, the SFM + IBMX was removed from each well and fresh SFM + IBMX media containing GLP-1/GLP-2 (GLP-1, 7-36,amide from Sigma, [Gly2]hGLP-2 from Allelix) concentration 1,3, 10 and 30nM were added to the appropriate wells. Plates incubated at 37C incubator for 30 min. Following incubation, the media were removed from each well. The wells were washed once with 1ml PBS(Phosphate Buffered Saline). Each well was then treated with 1ml cold 95% ethanol: 5mM EDTA (2:1) at 4C for 1 hr. Cells from each well then were scraped and transferred into individual eppendorf tubes. Tubes were centrifuged for 5 min at 4C, and the supernatants were transferred to new eppendorf tubes and dried in speed vacuum. Following drying tubes were reconstituted in 100ul of Na- Acetate and kept at 4C, 25 ul of this solution used for cAMP Assay.

The functional assay was performed as follows: cAMP content for each extract was determined in duplicate by EIA (Enzyme ImmunoAssay) using the Amersham Biotrak cAMP EIA Kit (Amersham 225).

Results of the assay are illustrated in Figure 3, which demonstrates the GLP-2 selectivity exhibited by the cloned receptor. In a similar assay of binding to the GLP-1 receptor, the expect pattern of binding preferentially to GLP-1 was observed.

SECRET 4224480

We claim:

1. An isolated nucleic acid encoding a GLP-2 receptor.
2. An isolated nucleic acid according to claim 1, which codes for a GLP-2 receptor having SEQ ID No.1.
3. An isolated nucleic acid according to claim 2, having SEQ ID No.2.
4. An isolated nucleic acid having a sequence that is at least 40% homologous with SEQ ID No.2
5. An isolated nucleic acid, characterized by:
(1) the ability to be amplified by PCR primers of SEQ ID No. 3 and SEQ ID No.4; and
(2) the ability to bind a probe of SEQ ID No. 5.
6. A recombinant DNA molecule comprising a nucleic acid encoding a GLP-2 receptor, and expression controlling elements linked operably with said nucleic acid to drive expression thereof
7. A recombinant DNA molecule according to claim 6, wherein said nucleic acid encodes a GLP-2 receptor of SEQ ID No.1.
8. A recombinant DNA molecule according to claim 7, adapted for expression in a mammalian cell.
9. A cell that has been adapted by genetically by the insertion of nucleic acid coding for a GLP-2 receptor.
10. A cell according to claim 9, wherein said cell is adapted by insertion of a recombinant DNA molecule in which nucleic acid coding for an GLP 2 receptor and expression

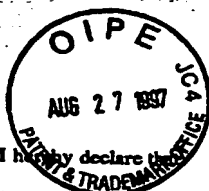
controlling elements functional in said cell are linked operably to drive expression of said nucleic acid.

11. A cell according to claim 10, wherein said nucleic acid encodes a GLP-2 receptor of SEQ ID No. 1.
12. A cell according to claim 11, wherein said nucleic acid is one having SEQ ID No. 2
13. A nucleic acid probe of at least 15 nucleotides, capable of specifically hybridizing with a unique region of a nucleic acid encoding a GLP-2 receptor.
14. An antisense oligonucleotide having a sequence capable of hybridizing to mRNA encoding a GLP-2 receptor so as to prevent translation of the mRNA.
15. A GLP-2 receptor antibody.
16. A transgenic non-human animal, having incorporated expressibly therein a transgene which codes for a GLP-2 receptor.
17. A transgenic non-human animal comprising a homologous recombination knockout of DNA coding for the GLP-2 receptor native to said animal.
18. A transgenic non human animal which incorporates and expresses antisense DNA as defined in claim 14.
19. A recombinant GLP 2 receptor.
20. The GLP 2 receptor of claim 19, having SEQ. ID No. 2
21. A method for identifying GLP 2 receptor ligands, which comprises the steps of

- (1) incubating a test ligand with a cell as defined in claim 9 or with a membrane preparation obtained therefrom; and then
 - (2) determining the extent of binding between the GLP 2 receptor and the test ligand.
22. A method for identifying GLP 2 receptor ligands, which comprises the steps of
- (1) incubating a test ligand with a cell as defined in claim 10 or with a membrane preparation obtained therefrom; and then
 - (2) determining the extent of binding between the GLP 2 receptor and the test ligand.
23. A GLP-2 receptor ligand identified by the method of claim 21.

The invention relates to nucleotides and amino acid sequences encoding glucagon-like peptide 2 receptors, recombinant host cells transformed with such nucleotides, and methods of using the same in drug screening and related applications.

5



#3

PENNIE & EDMONDS LLP DOCKET NO. 8607-011-999

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that
My residence, post office address and citizenship are as stated below at 201 et seq. underneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

CLONED GLUCAGON-LIKE PEPTIDE 2 RECEPTORS

and for which a patent application:

- ☐ is attached hereto and includes amendment(s) filed on _____ (if applicable)
☒ was filed in the United States on December 13, 1996 as Application Serial No. 08/767,224 (for declaration not accompanying application)
with amendment(s) filed on _____ (if applicable)
☐ was filed as PCT international application Serial No. _____ on _____ and was amended under PCT Article 19 on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES <input type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

POWER OF ATTORNEY: As a named inventor, I hereby appoint S. Leslie Misrock (Reg. No. 18872), Harry C. Jones, III (Reg. No. 20280), Berj A. Terzian (Reg. No. 20060), Gerald J. Flintoft (Reg. No. 20823), David Weild, III (Reg. No. 21094), Jonathan A. Marshall (Reg. No. 24614), Barry D. Reia (Reg. No. 22411), Stanton T. Lawrence, III (Reg. No. 25736), Isaac Jarkovsky (Reg. No. 22713), Joseph V. Colaianni (Reg. No. 20019), Charles E. McKenney (Reg. No. 22795), Philip T. Shannon (Reg. No. 24278), Francis E. Morris (Reg. No. 24615), Charles E. Miller (Reg. No. 24576), Gidon D. Stern (Reg. No. 27469), John J. Lauter, Jr. (Reg. No. 27814), Brian M. Poissant (Reg. No. 28462), Brian D. Coggio (Reg. No. 27624), Rory J. Radding (Reg. No. 28749), Stephen J. Harbulak (Reg. No. 29166), Donald J. Goodell (Reg. No. 19766), James N. Palik (Reg. No. 25510), Thomas E. Friebe (Reg. No. 29258), Laura A. Coruzzi (Reg. No. 30742), Jennifer Gordon (Reg. No. 30753), Jon R. Stark (Reg. No. 30111), Allan A. Fanucci (Reg. No. 30256), Geraldine F. Baldwin (Reg. No. 31232), Victor N. Balancia (Reg. No. 31231), Albert P. Halluin (Reg. No. 25227), Samuel B. Abrams (Reg. No. 30605), Steven I. Wallach (Reg. No. 35402), Marcia H. Sundeen (Reg. No. 30893), Paul J. Zegger (Reg. No. 33821), Edmond R. Bammon (Reg. No. 32110), Bruce J. Barker (Reg. No. 33291), Adriano M. Antler (Reg. No. 32605), Ann L. Gisolfi (Reg. No. 31956), SaraLynn Mandel (Reg. No. 31853), Mark A. Farley (Reg. No. 33170), James G. Markey (Reg. No. 31636), and Charles F. Hoyne (Reg. No. 35548), all of Pennie & Edmonds LLP, whose addresses are 1155 Avenue of the Americas, New York, New York 10036, 1667 K Street N.W., Washington, DC 20006 and 3300 Hillview Avenue, Palo Alto, CA 94304-1203, and each of them, my attorneys, to prosecute this application, and to transact all business in the Patent and Trademark Office connected therewith.

SEND CORRESPONDENCE TO: PENNIE & EDMONDS LLP 1155 AVENUE OF THE AMERICAS NEW YORK, N.Y. 10036-2711			DIRECT TELEPHONE CALLS TO: PENNIE & EDMONDS LLP DOCKETING (212) 790-2803	
201	FULL NAME OF INVENTOR	LAST NAME <u>Munroe</u>	FIRST NAME <u>Donald</u>	MIDDLE NAME <u>G</u>
	RESIDENCE & CITIZENSHIP	CITY <u>Waterdown</u>	STATE OR FOREIGN COUNTRY <u>Ontario, Canada</u> CAX	COUNTRY OF CITIZENSHIP <u>Canada</u>
	POST OFFICE ADDRESS	STREET <u>27 Wakefield Lane</u>	CITY <u>Waterdown</u>	STATE OR COUNTRY <u>Ontario, Canada</u> ZIP CODE <u>LOR 2H3</u>
202	FULL NAME OF INVENTOR	LAST NAME <u>Gupta</u>	FIRST NAME <u>Ashwani</u>	MIDDLE NAME <u>K</u>
	RESIDENCE & CITIZENSHIP	CITY <u>Mississauga</u>	STATE OR FOREIGN COUNTRY <u>Ontario, Canada</u> CAX	COUNTRY OF CITIZENSHIP <u>Canada</u>
	POST OFFICE ADDRESS	STREET <u>7031 Dunrobin Way</u>	CITY <u>Mississauga</u>	STATE OR COUNTRY <u>Ontario, Canada</u> ZIP CODE <u>LSN 6Y4</u>
203	FULL NAME OF INVENTOR	LAST NAME <u>Vyas</u>	FIRST NAME <u>Tejal</u>	MIDDLE NAME <u>B</u>
	RESIDENCE & CITIZENSHIP	CITY <u>Mississauga</u>	STATE OR FOREIGN COUNTRY <u>Ontario, Canada</u> CAX	COUNTRY OF CITIZENSHIP <u>Canada</u>
	POST OFFICE ADDRESS	STREET <u>275 Riel Drive</u>	CITY <u>Mississauga</u>	STATE OR COUNTRY <u>Ontario, Canada</u> ZIP CODE <u>LSB 3K1</u>
204	FULL NAME OF INVENTOR	LAST NAME <u>McCallum</u>	FIRST NAME <u>Kirk</u>	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY <u>Mississauga</u>	STATE OR FOREIGN COUNTRY <u>Ontario, Canada</u> CAX	COUNTRY OF CITIZENSHIP <u>Canada</u>
	POST OFFICE ADDRESS	STREET <u>3618 Pitch Pine Crescent</u>	CITY <u>Mississauga</u>	STATE OR COUNTRY <u>Ontario, Canada</u> ZIP CODE <u>L5L 1P8</u>
205	FULL NAME OF INVENTOR	LAST NAME <u>Fan</u>	FIRST NAME <u>Ermei</u>	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY <u>Toronto</u>	STATE OR FOREIGN COUNTRY <u>Ontario, Canada</u> CAX	COUNTRY OF CITIZENSHIP <u>Canada</u>
	POST OFFICE ADDRESS	STREET <u>20 Southport Street, Apt. #418</u>	CITY <u>Toronto</u>	STATE OR COUNTRY <u>Ontario, Canada</u> ZIP CODE <u>M6S 4Y8</u>
206	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY ZIP CODE

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201 <u>Donald G. Munroe</u> DATE <u>Aug 7 1997</u>	SIGNATURE OF INVENTOR 202 <u>Ashwani K. Gupta</u> DATE <u>AUG 7, 1997</u>	SIGNATURE OF INVENTOR 203 <u>Tejal B. Vyas</u> DATE <u>Aug 7, 1997</u>
SIGNATURE OF INVENTOR 204 <u>Kirk McCallum</u> DATE <u>Aug 7 1997</u>	SIGNATURE OF INVENTOR 205 <u>Ermei Fan</u> DATE <u>AUG 7, 1997</u>	SIGNATURE OF INVENTOR 206 DATE

#3
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: ☒ Application of: Monroe, *et al.*
☐ Patent of:



☒ Serial No.: 08/767,224
☐ Patent No.:
☒ Filed: December 13, 1996
☐ Issued:

Group Art Unit: 1804

Examiner: N/A

For: CLONED GLUCAGON-LIKE PEPTIDE 2 RECEPTORS
Attorney Docket No.: 8607-011-999

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
[37 CFR 1.9(f) and 1.27(c)] - Small Business Concern

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I hereby declare that I am

- ☐ the owner of the small business concern identified below:
☒ an official of the small business concern empowered to act in behalf of the concern identified below:

Name of concern Allelix Biopharmaceuticals Inc.

Address of concern 6850 Goreway Drive

Mississauga, Ontario, Canada L4V 1V7

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the person employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern and/or there is an obligation under contract or law by the inventor(s) to convey rights to the small business concern with regard to the invention, entitled CLONED GLUCAGON-LIKE PEPTIDE 2 RECEPTORS by inventor(s) Donald ~~Mc~~ Monroe, Ashwani K. Gupta, Tejal B. Vyas, Kirk McCallum and Ermei Fan described in

- ☐ the specification filed herewith
☒ application serial no. 08/767,224 filed December 13, 1996
☐ patent no. issued

- If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

FULL NAME _____
ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

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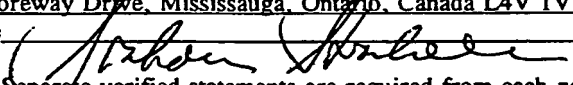
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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28 (b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, and patent issuing thereon, or any patent to which this verified statement is directed.

Send correspondence to: PENNIE & EDMONDS LLP Direct Telephone calls to:
1155 Avenue of the Americas PENNIE & EDMONDS LLP
New York, N.Y. 10036-2711 (212) 790-9090

Name of person signing Graham Strachan
Title of person other than owner President and Chief Executive Officer
Address of person signing Allelix Biopharmaceuticals Inc.
6850 Goreway Drive, Mississauga, Ontario, Canada L4V 1V7
Signature  Date June 16/97

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities.
(37 CFR 1.27)

1 TCTCCACTCC CAACAGATGC GTCTGCTGTG GGGCCCTGGG AGGCCCTTCC
51 TCGCCCTGCT TCTGCTGGTT TCCATCAAGC AAGTTACAGG ATCGCTCCTC
101 AAGGAGACAA CTCAGAAGTG GGCTAATTAT AAGGAGAAGT GTCTGGAAGA
151 CTTGCACAAT AGACTTTCTG GCATATTTTG TAATGGGACA TTTGATCGGT
201 ATGTGTGCTG GCCTCATTCT TATCCTGGAA ATGTCTCTGT TCCCTGTCCT
251 TCATACTTAC CTTGGTGGAA TGCAGAGAGC CCAGGAAGGG CCTACAGACA
301 CTGCTTGGCT CAGGGGACTT GGCAGACGCG AGAGAACACC ACAGATATTT
351 GGCAGGATGA ATCAGAATGC TCAGAGAACC ACAGCTTCAG ACAAACGTG
401 GATCACTACG CTTTGCTATA CACCTTGCAG CTGATGTACA CTGTGGGCTA
451 CTCCGTGTCT CTCATCTCCC TCTTCTTGGC TCTTACACTC TTCTTGTTCC
501 TTCGAAACT GCATTGCACA CGCAATTACA TCCACATGAA CCTGTTGCT
551 TCGTTCATCC TGAAAGTTCT GGCTGTCCTG GTGAAGGACA TGGTCTCCCA
601 CAACTCTTAC TCCAAGAGGC CCGATGATGA GAGTGGATGG ATGTCATATC
651 TGTCAGAGAC ATCCGTCTCC TGTGCTCCG TCCAGGTCCT CCTGCACTAC
701 TTTGTGGGCA CCAATCACTT GTGGCTGCTG GTTGAAGGAC TTTACCTCCA
751 CACTCTGCTG GAGCCACAG TGTTCCTGA AAGGCGGCTG TGGCCCAAGT
801 ACCTGGTGGT GGGTTGGGCC TTCCCCATGC TGTTCGTTAT TCCCTGGGGT
851 TTTGCCCCGTG CACACCTGGA GAACACACGG TGCTGGGCCA CAAATGGGAA
901 CCTGAAAATC TGGTGGATCA TCAGAGGACC CATGCTGCTT TGTGTAACAG
951 TTAATTTCTT CATCTTCCTC AAGATTCTCA AGCTTCTCAT TTCTAAGCTC
1001 AAAGCTCATC AGATGTGCTT CAGAGACTAC AAATACAGAT TGGCGAAATC
1051 AACGTTGCTC CTCATTCCTT TGTGGGGGT TCATGAGGTC CTCTTCACTT
1101 TCTTCCCCGA CGACCAAGTT CAAGGATTTT CAAAACGTAT TCGACTCTTC
1151 ATCCAGCTGA CACTGAGCTC TGTCCACGGA TTTCTGGTGG CCTTGCACTA

FIGURE 1

08/767224

2/4

1201 TGGCTTTGCC AATGGAGAGG TGAAGGCAGA GCTGCGAAAG TCATGGGGCC
1251 GCTTCTTATT AGCCCGCCAC TGGGGCTGCA GAACCTGTGT CCTGGGGAAG
1301 AATTTCCGGT TCCTGGGGAA GTGTTCCAAG AAGCTGTCGG AGGGAGATGG
1351 CTCTGAGACA CTCCAGAAGC TGCGGTTCTC CACATGCAGC TCACACCTGG
1401 CCTCTGAGAC CCTGGGAGAC GTTGGGGTAC AGCCTCACAG GGGCCGTGGA
1451 GCTTGGCCCC GGGGAAGCAG CCTGTCTGAG AGCAGTGAGG GAGACTTCAC
1501 CCTGGCCAAT ACGATGGAGG AGATTCTGGA AGAGAGTGAG ATCTAAGGCA
1551 GGGTCCATCA CCGCAGCTTG GCCA

FIGURE 1A

00767224.1300

1 MRLWGPGRP FLALLLVSI KQVTGSLKE TTQKWANYKE KCLEDLHNRL

51 SGIFCNGTFD RYVCWPHSYP GNVSVPCPSY LPWWNAESPG RAYRECLAQG
TM-1

101 TWQTRENTTD IWQDESECSE NHSFRQNDH YALLYTLOLM YTVGYSVSLI
TM-2

151 SLFLALTFL FLRKLCNTRN YIHMLFASF ILKVLAVLVK DMVSHNSYSK
TM-3

201 RPDDSGWMS YLSETSVSCR SVQVLLHYFV GTNHLWLLVE GLYLTLLLEP
TM-4

251 TVFPERRLP KYLVVGWAFP MLFVIPWGFA RAHLENTRCW ATNGNLKIWW
TM-5 TM-6

301 IIRGPMILCV TVNFFIFLKI IKLLISKKA HQMCFRDYKY RLAKSTLLLI
TM-7

351 PLLGVHEVLF TFFEDDOVQG FSKRIRLFIO LTLSSVHGFL VALQYGFANG

401 EVKAELRKSW GRFLLARHWG CRTCVLGKNF RFLGKCSKKL SEGDGSETLO

451 KLRFTCSSH LASETLGDVG VQPHRGRGAW PRGSSLSSESS EGDFTLANTM

501 EEILEESEI*

FIGURE 2

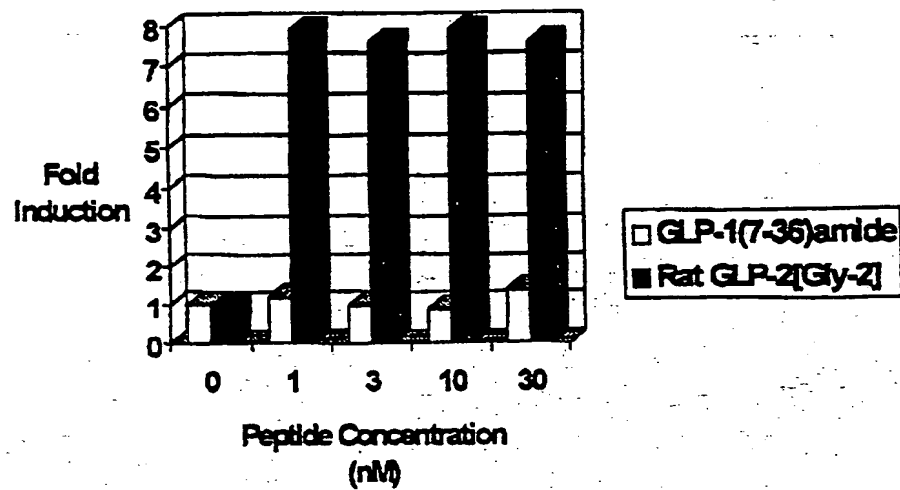
cAMP Response of COS Transfected with Clone 587

FIGURE 3

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